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Overcoming sodium toxicity by utilizing grass leaves as co-substrate during the start-up of batch thermophilic anaerobic digestion

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Abstract

Sodium toxicity is a common problem causing inhibition of anaerobic digestion, and digesters treating highly concentrated wastes, such as food and municipal solid waste, and concentrated animal manure, are likely to suffer from partial or complete inhibition of methane -producing consortia, including methanogens. When grass clippings were added at the onset of anaerobic digestion of acetate containing a sodium concentration of 7.8 g Na^+/L, a total methane production about 8 L/L was obtained, whereas no methane was produced in the absence of grass leaves. In an attempt to narrow down which components of grass leaves caused decrease of sodium toxicity, different hypotheses were tested. Results revealed that betaine could be a significant compound in grass leaves causing reduction to sodium inhibition.

Keywords: Sodium toxicity, grass leaves, thermophilic anaerobic digestion, start-up, betaine

1. Introduction

Anaerobic digestion of wastes, in particular solid wastes, results in the solubilization of organic and inorganic salts. The inhibitory effect of accumulating salts on the methanogenic microbiota in the anaerobic digester is a problem that is not well understood. One of the ions that always accumulates and that has been shown to be toxic to methanogenic Archaea is sodium.

Although sodium is essential for bacterial growth (Dimroth and Thomer, 1989), high sodium concentrations increase osmotic stress that can result in decreased cell activity and cell plasmolysis (Uygur, 2006). The occurrence of high sodium concentrations in an anaerobic reactor can generally be attributed to a high sodium concentration in the influent waste stream or sodium addition during operation of the digestion process. As a result of using sodium salts as an additive in a unit process such as food or bio-diesel production, high in sodium concentrations are generated. Industries, such as the seafood processing industries, utilize raw materials containing high sodium salts resulting in the generation of a salty wastewater. High sodium concentrations in an anaerobic digester can also arise from the addition of alkaline solution in the form of sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) or sodium bi-carbonate ($NaHCO_3$) to neutralize acidity during start-up and operation.

Although anaerobic digestion of saline wastewaters such as effluents from tannery industries (Lefebvre et al., 2006), seafood-processing (Omil et al., 1995) and oil and gas production (Ji et al., 2009) have been studied, solutions to the problem of inhibitory high sodium salts are still limited. One way of tackling the sodium salts problem, is by allowing the anaerobic sludge to acclimate to high sodium concentrations (Vyrides et al., 2009), but this technique requires time for the methanogens to adapt to the saline conditions which in turn results in a prolonged period

before the anaerobic reactor can achieve its full-loading capacity. Mendez et al. (1995) stated that a start-up period of nine months was required for the adaptation of anaerobic sludge to effectively treat saline seafood-processing wastewater. The use of halophilic methanogens as an inoculum has also been reported as an approach to deal with high sodium salts problems (Riffat and Krongthamchat, 2007). However, in a practical sense, it may be difficult to obtain halophilic methanogens for anaerobic reactors located far from the sea. One possible organic compound, which can cause antagonism against sodium toxicity, is glycine-betaine (GB) (Yerkes et al., 1997; Vyrides et al., 2010). GB ($(CH_3)_3N^+CH_2COO^-$), also known as betaine, is a trimethylated derivative of glycine (Rudulier and Bouillard, 1983). GB is one of the "compatible solutes" involved in osmoregulation at high osmotic pressure in many plants (Oishi and Ebina, 2005) and halophilic methanogens (Robertson et al. 1990; Lai and Gunsalus, 1992). Compatible solutes are soluble organic compounds, not involved in normal cell metabolism (Yerkes et al., 1997), although high concentrations of these solutes are accumulated within bacterial cells that are under salt stress. However, using GB to decrease sodium toxicity in commercial-scale anaerobic digesters would be too costly.

This study originated from observations that thermophilic anaerobic digestion can be started up successfully in the presence of high sodium bicarbonate concentrations (330 mM) added to avoid early build-up of volatile fatty acids (Suwannoppadol et al., 2011). It therefore appears that factors present in this type of digestions mitigate the usually observed inhibition by salt. To test if plant material (grass leaves) contributes to overcoming salt inhibition, thermophilic digestion of acetate was carried out with turf soil as source of methanogens and grass leaves as co-substrate. Moreover, compounds present in grass leaves such as betaine and potassium were investigated as a possible cause of overcoming sodium toxicity.

2. Materials and Methods

2.1 Inoculum sources and grass leaves

Turf soil samples were used as source of methanogens (Suwannoppadol et al., 2012). Leaves and soil were collected from a grassy area at Murdoch University, Perth, Australia. After removing grass leaves and the main grass roots the soils were used immediately as thermophilic anaerobic inoculum. All experiments were carried out in 100 mL serum vials with 10 or 15 g of turf soil and 40 or 50 mL of culture medium described in section 2.2. One-hundred g/L of fresh grass leaves was added to test for reduction of sodium toxicity where applicable. For mesophilic tests, 40 mL of mesophilic anaerobic sludge (soluble chemical oxygen demand =1541 mg/L, total solids = 32.3 g/L, total suspended solids = 29.0 g/L, volatile solids = 24.3 g/L, and volatile suspended solids = 24.2 g/L) was used as inoculum and the final working volume was adjusted to 60 mL with culture medium. Mesophilic anaerobic sludge was collected from the Woodman Point Wastewater Treatment Plant treating municipal wastewater located in Perth, Western, Australia.

2.2 Culture medium composition and carbon source

To adjust the working volume of serum vial, culture medium, sterilized grass juice (chemical oxygen demand 3331 mg/L or filtered grass juice were used. When necessary, the pH of culture medium was adjusted to 7.5 ± 0.2 with 1 M HCl.

The culture medium contained (per liter): 0.3 g KH_2PO_4, 0.6 g NaCl, 0.1 g $MgCl_2.2H_2O$, 0.08 g $CaCl_2.2H_2O$, 1.0 g NH_4Cl, 3.5 g $KHCO_3$, 10 mL of vitamin solution, and 5 mL of trace element solution. Vitamin solution contained (per liter): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg

pyridoxine hydrochloride, 5.0 mg thiamin hydrochloride, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg DL-calcium pantothenate, 0.1 mg vitamin B_{12}, 5.0 mg *p*-aminobenzoate, and 5.0 mg lipoic acid. Trace element solution contained (per liter): 12.8 g nitrilotriacetic acid, 1.35 g $FeCl_3.6H_2O$, 0.1 g $MnCl._4H_2O$, 0.024 g $CoCl_2.6H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1 g $ZnCl_2$, 0.025 g $CuCl_2.2H_2O$, 0.01 g H_3BO_3, 0.024 g $Na_2MoO_4.4H_2O$, 1.0 g NaCl, 0.12 g $NiCl_2.6H_2O$, 4.0 mg $Na_2SeO_3.5H_2O$, 4.0 mg $Na_2WO_4.2H_2O$.

2.3 Preparation of sterilized grass leaves, sterilized grass juice, filtered grass juice, and ash from grass leaves

Mix-species of grass leaves were collected at least 2 cm above the soil profile to minimize contamination by the soil. To prepare sterilized grass leaves, 5 g of grass leaves were autoclaved at 120 °C for 40 min.

To prepare sterilized grass juice (chemical oxygen demand = 3331 mg/L), 5 g of fresh grass leaves and 50 mL of culture medium were blended with a mechanical blender (DēLonghi, model DBL740) for 15 min. The grass residue was removed from the grass juice by filtering through cloth with pore size of 1 mm and the filtrate was sterilized by autoclaving at 120 °C for 40 min or by filtration through a 0.2 μm filter paper (Whatman).

To prepare a solution of ash from grass leaves, 5 g of grass leaves was combusted in a furnace at 550 °C for 24 h. The ash was dissolved in 50 mL of culture medium and neutralized by addition of 1M HCl.

2.4 Experimental design

Experiments were conducted in duplicate 100 mL serum vials (Wheaton) sealed with butyl rubber stoppers and aluminum crimps at 55 ˚C or the mesophilic experiment at 37 ˚C. To establish anaerobic conditions, the headspaces of all serum vials were flushed with N_2/CO_2 (80%/20%) for 30 seconds. All samples were incubated in a water bath (Paton, model RW 1812) with shaking (30 oscillations/min). All serum vials were depressurized to atmospheric pressure after the first hour of incubation. The volume of biogas produced was measured using a 50 mL glass syringe (Popper & Sons, Inc.).

2.5 Analysis

For VFA analysis, 0.25 mL of liquid sample was removed via syringe through the rubber seal of the serum test vials. The supernatant was centrifuged at 12,000 rpm for 5 min (Hermle Z233M2) to obtain a clear solution. The supernatant was used for VFA analysis or stored at -20 ˚C. The VFA concentrations of samples were analyzed by gas chromatography (GC) using a Varian Star 3400 equipped with a Varian 8100 auto sampler and a flame ionization detector (FID) as described by Walker et al. (2009).

The methane concentration in biogas was analyzed with a Varian Star 3400 gas chromatograph equipped with a thermal conductivity detector as described by Charles et al. (2009).

Betaine in grass leaves was analyzed by ChemCentre, Perth, Western Australia, a nationally accredited analytical laboratory. Betaine present in grass leaves was analyzed by using a combination of liquid chromatography and mass spectrometry (LC-MS). For grass extraction, 0.25 g of grass leaves sample was extracted with 10 mL of 50% methanol. After filtration, the extracted grass solution was transferred to HPLC for LC-MS analysis. HPLC was done with a

Zorbax Eclipse Plus C column (18 4.6 x 50 mm, 1.8 µm) and a pump system (Agilent 1260 Infinity Binary Pump System). The mobile phase consisted of 10 mM ammonium formate pH 3 (A) and acetonitrile (B) at an isocratic with 10% acetonitrile for 10 minutes. MS analyses were carried out on Triple Quad LCMS operating in electro-spray ionisation (ESI) mode. Analytical data was acquired in Multiple Reaction Monitoring (MRM) mode (precursor ion 118, product ion 59, positive ion mode). After the LC and MRM protocols were selected, standard curves were determined. Five different concentrations of betaine were prepared and analyzed by LC-MS/MS to generate the standard curves using linear regression lines.

Total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS) and chemical oxygen demand (COD) were analysed according to the American Public Health Association (2005).

2.6 Acetate and Sodium concentrations

Acetate, as carbon source, and sodium bicarbonate ($NaHCO_3$), as buffer and the main source of sodium ions, were added as dry salts after adjusting the working volume, but before degassing. To test the effect of elevated levels of sodium ions on thermophilic methane production, a concentration of 1.8 g Na^+/L resulting from using sodium acetate (80 mM) as carbon source (control), and a strongly inhibitory concentration (McCarty, 1964) of 7.8 g Na^+/L = 330 mM were selected (80 mM Na-acetate, 250 mM, NaHCO3, together resulting in a total of 7.8 g Na+/L). The pH of all tests was adjusted to 7.5 ± 0.2.

3. Results and Discussion

3.1 Effect of sodium concentration on methane production during the start-up of thermophilic anaerobic digestion

In the presence of 1.8 g Na^+/L, approximately 1.53 L/L of methane was produced, which is comparable to the expected theoretical methane production (1.9 L/L) from the acetate (80 mM) (Fig. 1). The spontaneous methane production at 1.8 g Na^+/L confirms the previously published observations that sufficient thermophilic acetate degrading methanogenic consortia are present in turf grass soil to enable a swift start-up of thermophilic anaerobic digestion (Suwannoppadol et al. 2011). Methane production and acetate degradation was completely inhibited in the presence of 330 mM (7.8 g Na^+/L).

3.2 Decrease of sodium toxicity in mesophilic and thermophilic anaerobic digestion by utilizing grass clippings

The addition of mix-species of grass clippings caused a five-fold increase in total methane produced (Fig. 2A) compared to the control that contained non-inhibitory sodium (1.8 g/L sodium) and no grass clippings (Fig. 2A). This increased methane production resulted from the grass clippings providing additional substrate for methane production (Fig. 2A). As expected, a test with just grass clippings and no acetate showed also high levels of methane formation (Fig. 2A).

In mesophilic experiments with grass leaves as a co-substrate , sodium toxicity on methanogenic acetate degradation was also reduced (Fig. 2C and 2D).

The fact that in the absence of grass clippings, more methane was formed than expected from acetate conversion alone can be explained by residual organics present in the inoculum (anaerobic digester sludge). Leaves of *Stenotaphrum secundatum*, *Cynodon dactylon*, and *Zoysia japonica* as co-substrates also enabled thermophilic methanogenesis in the presence of 7.8 g Na^+/L (Fig. 3).

The concept of co-digestion to decrease sodium toxicity during the anaerobic digestion of cow manure has already been described. Fang et al. (2011) studied the effects of co-digestion of de-sugared molasses (DM), containing high concentrations of sodium and potassium ions, with cow manure. The authors reported that methane yield from anaerobic digestion of a mixture 15% DM in cow manure was 190 ml-CH_4/gVS-added, which was approximately 50% higher than that of a mixture of 15% DM in water. Reasons as to which compounds in cow manure caused this effect were not given. The results of the current study point to the possibility that digested grass residue present in cow manure could play a role in the decrease of sodium toxicity in the above study.

3.3 Effects of sterilized grass juice and filtered grass juice on methane production in presence of high sodium concentration

Since fresh grass leaves contain significant numbers of thermophilic methanogens (Suwannoppadol et al., 2012), it was necessary to investigate whether the decrease in sodium inhibition was due to microorganisms on grass leaves or compounds within the leaves.

Again, at 7.8 g Na^+/L, there was no methane production when acetate was used as the sole carbon source , while in the presence of sterilized grass juice, significant methane was produced (2.5 L/L)(Fig. 4A) during three weeks of incubation. Filtered grass juice enabled acetate driven methanogenesis in the presence of sodium (7.8 Na^+/L)(Fig. 4A). This suggests that chemical species in the grass rather than microorganisms on the grass leaves were responsible for alleviating sodium toxicity.

3.4 Effects of potassium and ash from grass leaves on sodium inhibition in anaerobic digestion

To test whether the reduction in sodium toxicity imparted by grass juice was due to inorganic substances such as antagonistic cations (i.e. potassium), the ash of 5 g of grass leaves was tested and found to not overcome the described sodium inhibition of methanogenic acetate conversion (Fig. 5). Also potassium salt additions of 10, 25, and 50 mM were not able to counteract the sodium inhibition (80 mM) (Fig. 5).

It can be concluded that inorganic compounds in grass leaves and, in particular potassium, are not responsible for the antagonistic effects towards sodium toxicity in the current study. This is in disagreement with previous work reported by Kugelmam and McCarty (1964) who showed that adding potassium (6 g K^+/L) to an anaerobic digester, in the presence of 6 g Na^+/L, led to an increase in acetate degradation from 90 to 95%.

Müller et al. (2005) suggested that inorganic ions, mainly potassium are absorbed and accumulated in the cytoplasm of bacteria to balance the external osmotic pressure. However, the authors also stated that this mechanism required a long time period for the adaptation of intercellular enzymes to high salt concentrations. Vyrides et al. (2010), while examining the effect of potassium on the sodium toxicity of batch mesophilic anaerobic digestion at a sodium chloride concentration of 35 g/L, found that, after the addition of potassium ions, approximately one month was required for significant methane production to resume.

When testing for the minimum concentration of grass extract needed to overcome sodium toxicity, it was found that an extract concentration of 10% (extract of 5 g of grass in 50 mL of medium) had the full effect while the addition of 1% or 0.1% extract had no significant effect (data not shown).

3.5 Effects of glycine-betaine (GB) on sodium toxicity in thermophilic anaerobic digestion

Vyrides et al. (2010) investigated the effects of different GB concentrations (0.1 and 1 mM) on the decrease in sodium toxicity (35 g NaCl/L equivalent to about 13. 8 g Na^+/L) during batch anaerobic digestion using sewage sludge as an inoculum and found that the amount of methane produced were approximately three times higher when GB was added. GB is present in various plant species in particular halotolerant plants (Ashraf and Foolad, 2007) and has also been found in some turf grass species (Marcum and Murdoch, 1994).

Results showed that in the presence of 7.8 g Na^+/L sodium, methane was produced particularly in the presence of 10 mM glycine betaine (Fig. 6). As about 3.5 mol of methane can be formed per mol of glycine betaine ($(CH_3)_3N^+CH_2COO^-$), the anaerobic digestion of the 10 mM glycine betaine could potentially result in about 0.86 L/L of methane gas.

Previously, Yerkes et al. (1997) included GB in batch mesophilic anaerobic digestion seeded with enriched cultures of *Methanosarcina* (1850 mg/L) and *Methanothrix* (1350 mg/L) and found that GB at concentration 1 to 10 mM were effective in increasing methane production in the presence of sodium concentrations (about 17 g Na^+/L).

The analysis of the GB content of fresh grass leaves showed a GB content of 3.9 mg g^{-1}. This concentration would have provided a theoretical concentration of 3.3 mM in the digestion assays that included grass leaves. Such a concentration would have been in the range that resulted in a decrease of sodium toxicity.

4. Conclusion

Grass clippings are a cost-effective material for decreasing of sodium toxicity in mesophilic and thermophilic anaerobic digestions. Different chemical species in grass extract could contribute

to overcoming sodium toxicity and this study indicates that glycine-betaine could be one of them. Further research is needed to identify which other compounds in grass leaves help overcome sodium toxicity during anaerobic digestion.

Acknowledgements

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Figure captions

Figure 1: Effect of sodium concentration (1.8 (Δ) and 7.8 g Na^+/L, (▲)) on thermophilic methane production (A) and acetate conversion with 15 g/50 mL of turf soil as inoculum (B). Results are averages from duplicate tests (50 mL).

Figure 2: Effect of turf grass clippings and sodium ions on methane production and acetate degradation during thermophilic (A and B) and mesophilic (C and D) batch anaerobic digestion with 15 g/50 mL of turf soil as inoculum in duplicate serum vials. Additions: 80 mM of sodium acetate (1.8 g Na^+/L) (▲), 80 mM of sodium acetate + 250 mM of sodium bicarbonate (7.8 g Na^+/L) (○), 80 mM of sodium acetate + 250 mM of sodium bicarbonate + 5 g/50 mL of grass clippings (7.8 g Na^+/L) (■), and in the absence of sodium acetate but the presence of grass clippings (5 g/50 mL) with 330 mM of sodium bicarbonate (7.8 g Na^+/L) (□).

Figure 3: Effect of different turf grass species *Cynodon dactylon* (▲), *Zoysia japonica* (□), *Stenotaphrum secundatum* (♦) on methane production and acetate conversion (80 mM) during batch thermophilic anaerobic digestion with 10 g/40 mL of turf soil as inoculum in the presence of 7.8 g Na^+/L). Controls contain 100 g/L of grass leaves without acetate (■) and with acetate (80 mM) without grass leaves (○). All assays (40 mL) were in duplicate.

Figure 4: Effect of co-digestion of 80 mM acetate without (□) and with sterilized grass juice (○) or filtered grass juice (▲) on methane production (A) and acetate degradation (B) of batch thermophilic anaerobic digestion in the presence of 7.8 g Na^+/L in duplicate serum vials. 15 g/50 mL of turf soil served as the inoculum.

Figure 5: Effect of potassium chloride (10, 25, and 50 mM) (○), ash from 5 g of grass leaves (○) and 5 g of sterilized grass leaves (●) in the presence of 7.8 g Na^+/L on methane production during batch thermophilic anaerobic digestion with 15 g/50 mL of turf soil as inoculum in duplicate serum vials.

Figure 6: The effect of 1.8 g Na^+/L without betaine (x) as positive control and 7.8 g Na^+/L with betaine: 0 (○), 1 (□), 5 (Δ), and 10 mM (●) on methane production during the start-up period of batch thermophilic anaerobic digestion with 15 g/50 mL of turf soil as inoculum in duplicate serum vials.

Figures (1-6)

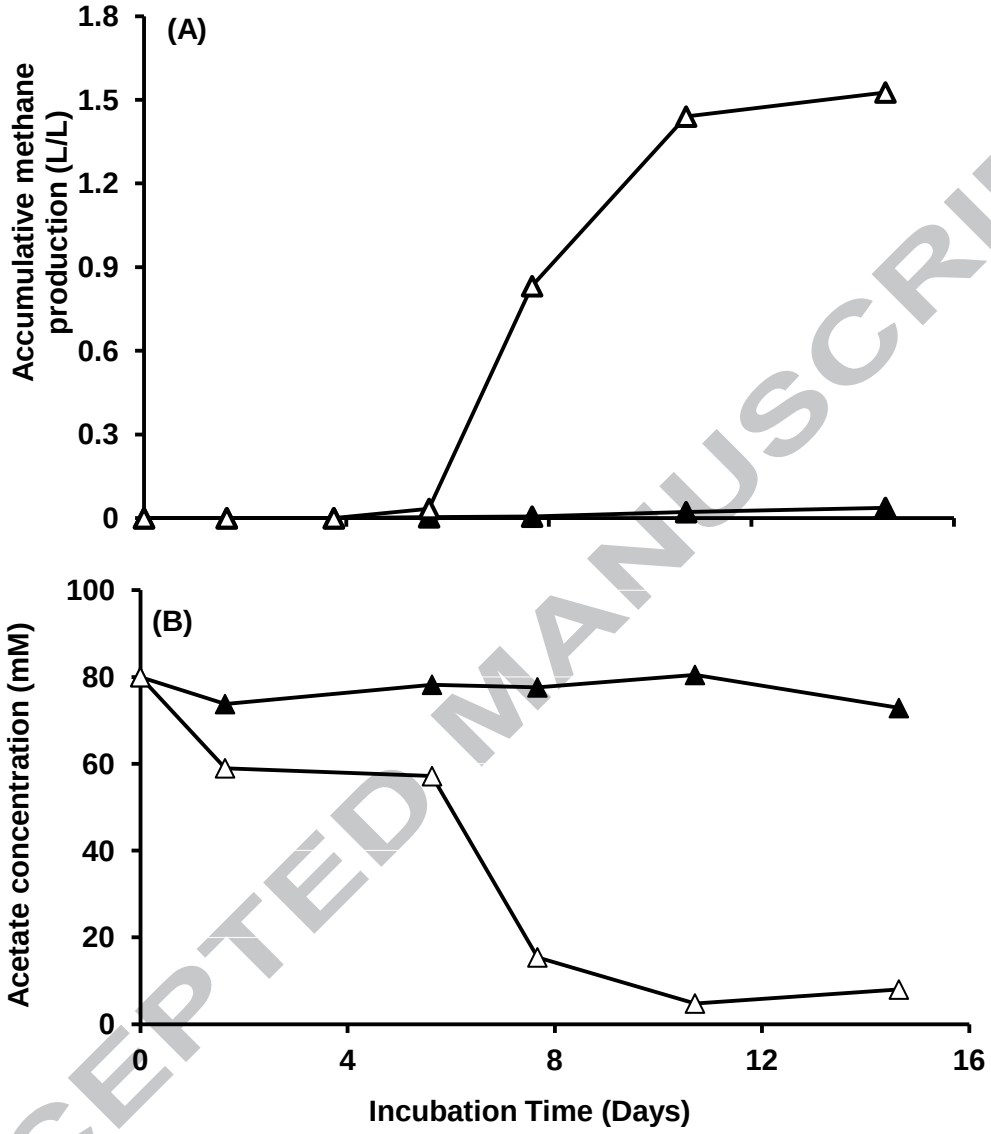


Figure 1

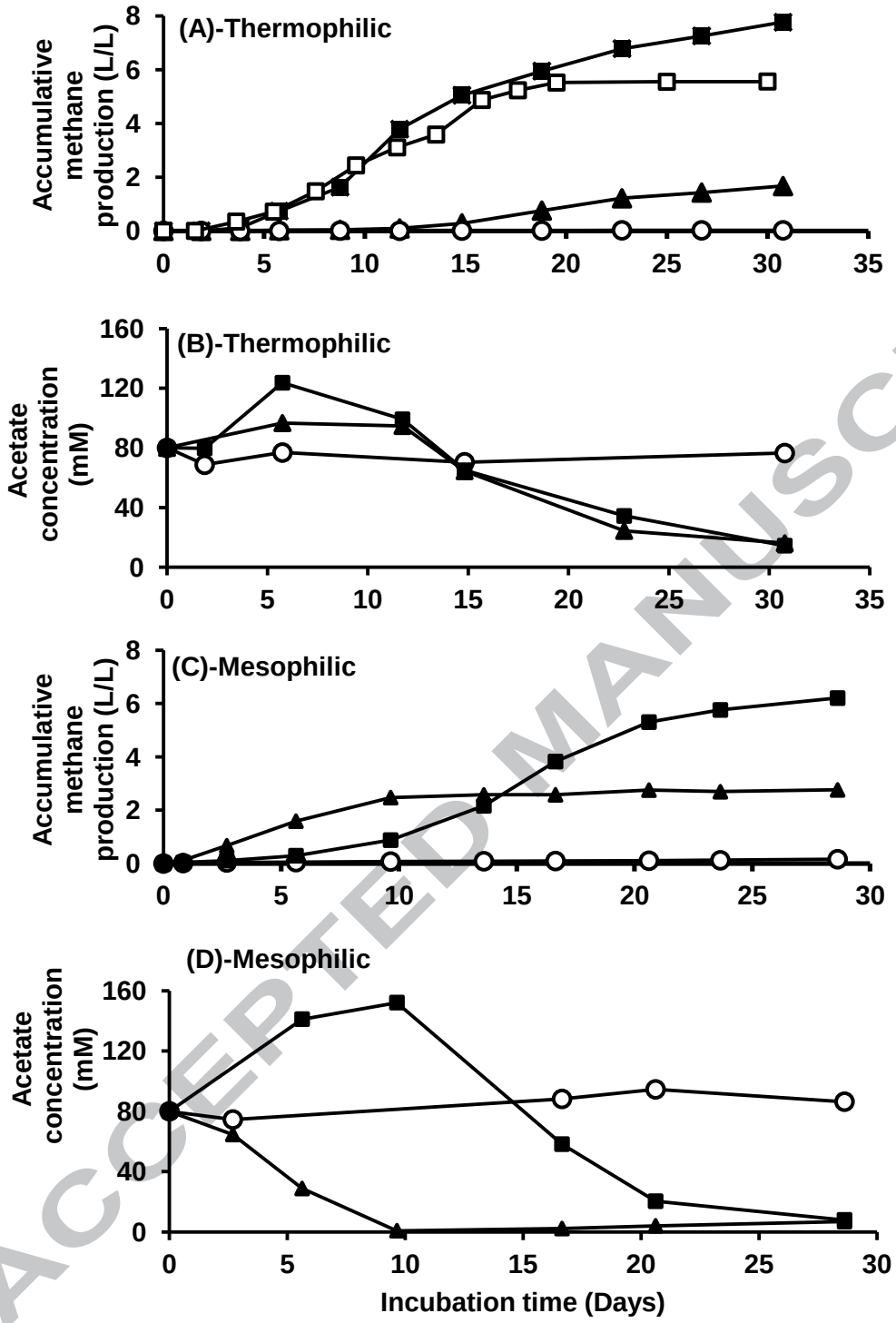


Figure 2

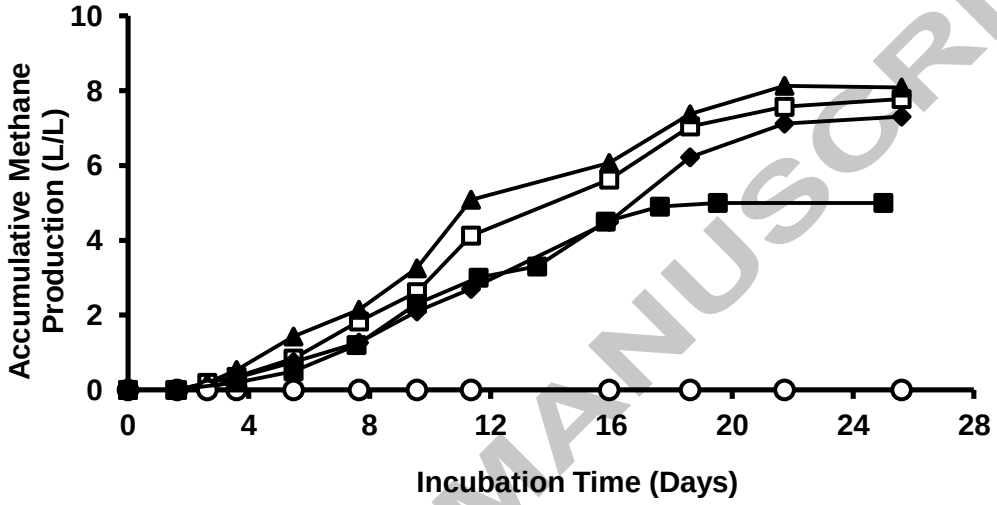
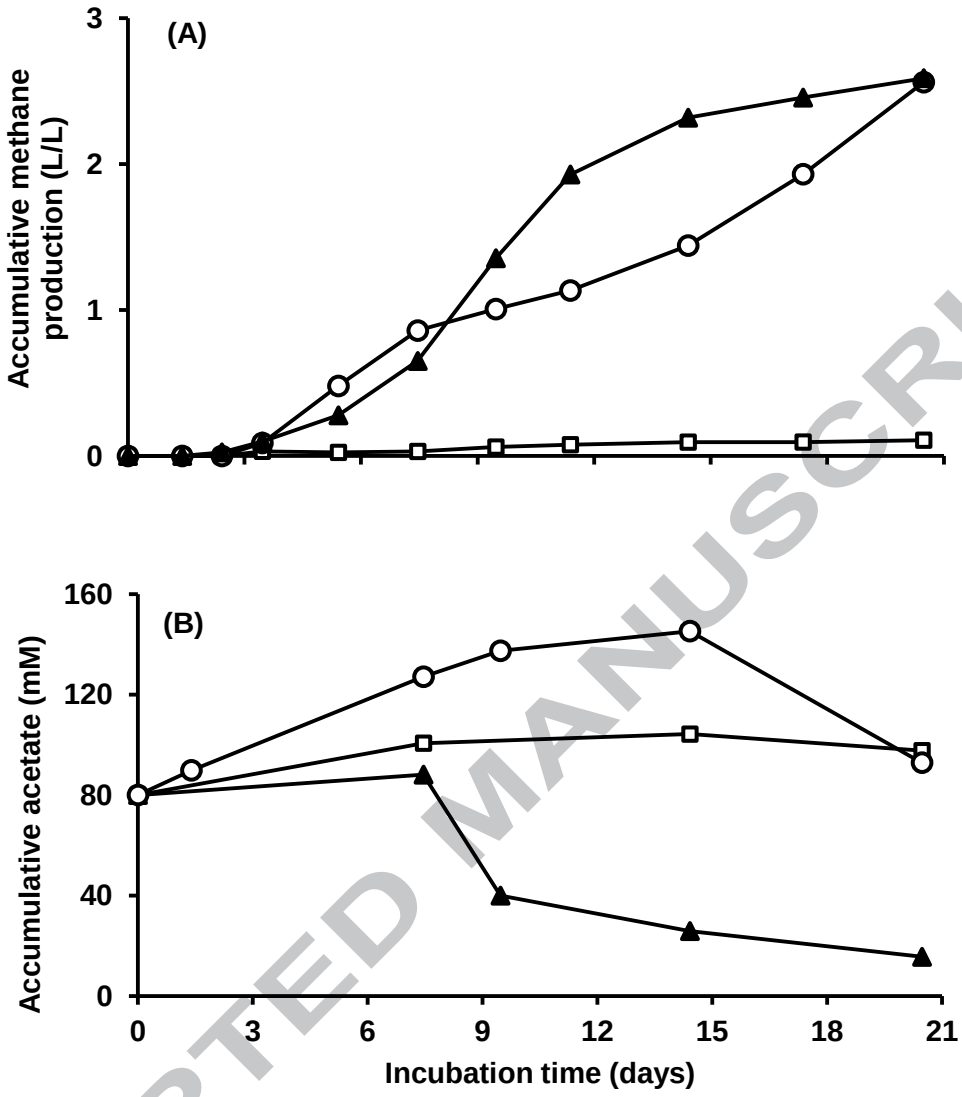


Figure 3



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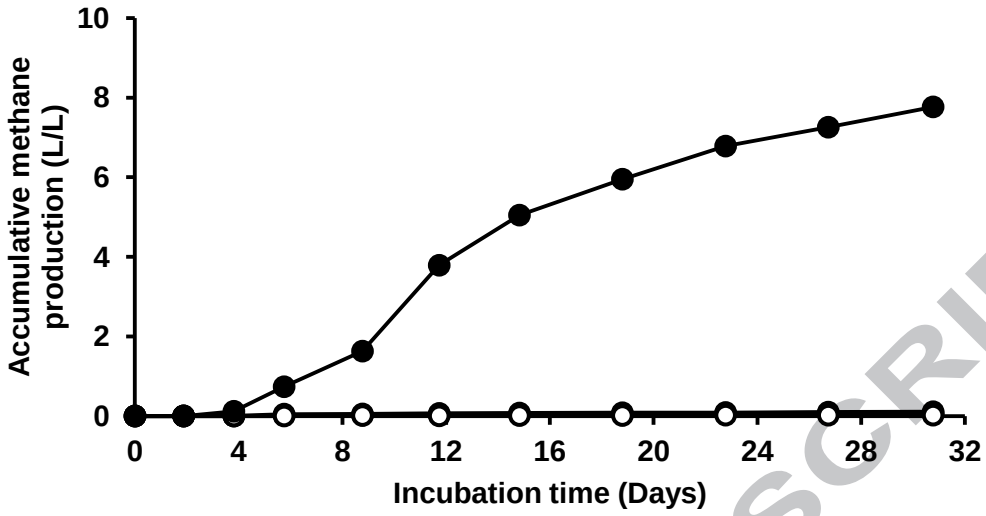

Figure 5

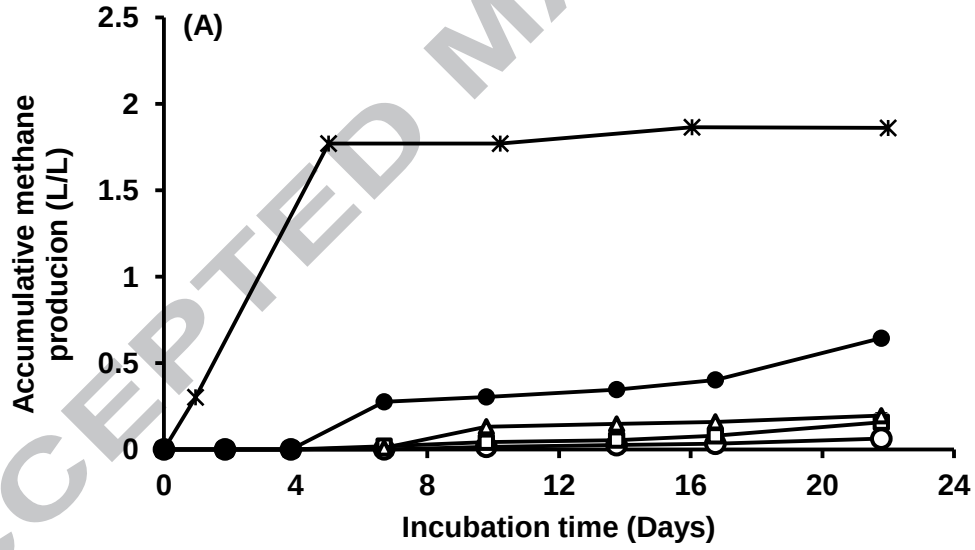

Figure 6

Highlights

- Sodium toxicity in anaerobic digestion can be overcome by adding grass clippings as co-substrate
- Different grass turf species can be used as co-substrate to decrease sodium toxicity
- Betaine could be a significant compound in grass causing reduction in sodium inhibition.